1: Biochem Biophys Res Commun. 1993 Mar 31;191 (3):1326-31.

A receptor-mediated antigen delivery and incorporation system. Administration of alpha 2-macroglobulin-cytochrome c conjugate induced high concentrations of antibodies against cytochrome c in mice.

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Specific receptors for alpha 2-macroglobulin (alpha 2M) are found on the plasma membrane of macrophages (M phi s), one of antigen presenting cells. So far, a receptor-mediated effective uptake by M phi of foreign antigens which were linked to alpha 2M has been shown to provoke a remarkable increase in the proliferation of T lymphocytes and in the production of antibodies in vitro. Such results encouraged us to develop a new type of vaccine using a receptor-mediated antigen delivery and incorporation system based on alpha 2M and its receptor interaction. In this report, we applied the system to experimental animals. Yeast cytochrome c was used as an antigen to see if the system worked in vivo as well as in vitro. Cytochrome c was conjugated to alpha 2M through the action of trypsin and intraperitoneally administered to mice. The titer induced in mice was measured by enzyme linked immunosorbent assay (ELISA). The production of antibodies against cytochrome c was significantly increased when the protein was given in conjugated forms with alpha 2M.

PMID: 7682069 [PubMed - indexed for MEDLINE]

Biochemistry

Volume 36, Number 41 October 14, 1997

Articles

Nickel Inhibits Binding of α_2-Macroglobulin-Methylamine to the Low-Density Lipoprotein Receptor-Related Protein/α_2-Macroglobulin Receptor but Not the α_2-Macroglobulin Signaling Receptor[†]

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Received April 8, 1997; Revised Manuscript Received June 30, 1997[®]

ABSTRACT: A previous study demonstrated that activated α_2-macroglobulin (α_2M*) binding to the low-density receptor-related protein/α_2-macroglobulin receptor (LRP/α_2MR) is blocked by Ni^{2+} [Hussain, M. M., et al. (1995) *Biochemistry 34*, 16074−16081]. We now report that the effect of Ni^{2+} is on a region of the α_2M molecule upstream of the carboxyl terminal receptor recognition domain. This observation is consistent with previous observations from this laboratory suggesting that α_2M* binding to LRP/α_2MR involves a region of the α_2M molecule immediately upstream of the receptor recognition domain [Enghild, J. J., et al. (1989) *Biochemistry 28*, 1406−1412]. We further demonstrate that Ni^{2+} has no effect on the binding of α_2M* or a cloned and expressed receptor binding fragment (RBF) to the recently described α_2M signaling receptor as assessed by direct binding and signal transduction studies.

The α-macroglobulins are part of a large super family including complement components C_3 and C_4 as well as human α_2-macroglobulin (α_2M)[1] and so called pregnancy zone protein [for reviews, see Sottrup-Jensen (1987) and Chu and Pizzo (1994)]. These proteins generally contain an internal β-cysteinyl-γ-glutamyl thiolester as well as a proteinase sensitive region. Proteolytic cleavage of human α_2M or direct nucleophilic attack on the thiolesters triggers a major conformational change which exposes receptor recognition sites present in each of the four α_2M subunits [for reviews, see Salvesen and Pizzo (1993) and Chu and Pizzo (1994)].

Two receptors bind α_2M*, namely, LRP/α_2MR and α_2-MSR. LRP/α_2MR is a scavenger receptor which binds a wide variety of ligands including α_2M*, RBF, lactoferrin, lipoprotein lipase, and *Pseudomonas* exotoxin A (Strickland et al., 1990; Kristensen et al., 1990) [for review, see Krieger and Herz (1994)]. RAP blocks binding of all known ligands to LRP/α_2MR; however, most of the other ligands which bind to this receptor appear to interact with independent domains and do not show cross-competition (Strickland et al., 1990; Krieger & Herz, 1994). Binding of α_2M* to LRP/α_2MR is followed by uptake and degradation in lysozomes, but not activation of a signaling cascade.

By contrast, binding of α_2M* or RBF to α_2MSR triggers classical signaling cascades as well as regulating cell proliferation (Misra et al., 1993; 1994a,b; 1995; 1997; Howard et al., 1996a,b; Webb et al., 1995). While RAP blocks binding of α_2M* and RBF to LRP/α_2MR, it has no effect on binding of these ligands to α_2MSR. The affinity of these ligands is also quite different being of extremely

† Supported by National Institutes of Health Grant HL-24066.

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® Abstract published in *Advance ACS Abstracts*, October 1, 1997.

[1] Abbreviations: α_2M, α_2-macroglobulin; α_2M*, activated α_2M; HHBSS, Hanks' balanced salt solution; LDL, low-density lipoprotein, LRP/α_2MR, the low-density lipoprotein receptor-related protein/α_2M receptor; RAP, receptor-associated protein; RBF, receptor binding fragment; Fura-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester; IP_3, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, the concentration of intracellular Ca^{2+}; *cis*-DDP, *cis*-dichlorodiammineplatinum(II).

S0006-2960(97)00806-4 CCC: $14.00

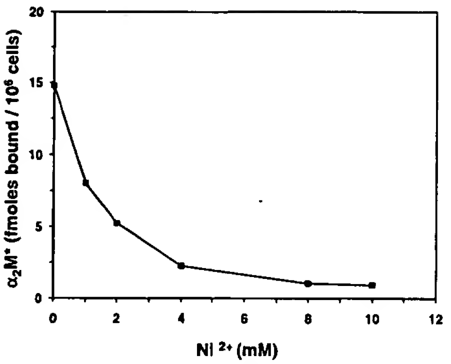

FIGURE 1: The effect of increasing concentrations of Ni^{2+} on the binding of α_2M^* to macrophages. The specific binding of [^{125}I]α_2M^* (0.5 nM) in the presence of increasing concentrations of Ni^{2+} was studied employing murine peritoneal macrophages which express both α_2M receptors, LRP/α_2MR and α_2MSR (Misra et al., 1994a,b; Howard et al., 1996a–c).

high affinity to the signaling receptor ($K_d \approx 50$ pM) and of moderately high affinity to the scavenger receptor ($K_d \approx 5$ nM).

Recently, Hussain et al. (1995) have reported that Ni^{2+} blocks binding of α_2M^* to LRP/α_2MR. These studies did not examine the effects of Ni^{2+} on the binding of α_2M^* to the signaling receptor. Moreover, these investigators did not study RBF binding to either receptor. In the present report, we demonstrate that Ni^{2+} blocks binding of α_2M^*, but not RBF, to LRP/α_2MR. Ni^{2+} did not affect binding of either ligand to α_2MSR and had no effect on the signaling cascade activated after α_2M^* or RBF binds to this receptor.

MATERIALS AND METHODS

Materials. α_2M was purified, characterized, activated with methylamine and radiolabeled with ^{125}I as previously described (Imber & Pizzo, 1981). This receptor-recognized form of α_2M is referred to as α_2M^* in the remainder of the text. The receptor binding fragment was cloned and expressed in *Escherichia coli*, characterized, and radiolabeled with ^{125}I as previously reported (Salvesen et al., 1992; Howard et al., 1996b,c). Fura-2/AM was purchased from Molecular Probes, Inc. (St. Louis, MO). 2-[^{3}H]*myo*-inositol (specific activity, 10–20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). RAP was prepared as previously described (Howard et al., 1996a). The pGEX 39 kDa expression construct was a kind gift of Dr. Joachim Herz (University of Texas, Southwestern, Dallas, TX). All other reagents were of the highest grade available.

Ligand Binding Assays. Thioglycolate-elicited macrophages were obtained from C57 Bl/6 mice (Charles Rivers Laboratories, Raleigh, NC) by peritoneal lavage as previously described (Misra et al., 1993, 1994a,b). The cells were plated in 48-well plates to achieve a density of 1×10^6 cells/well. The monolayers were washed three times in ice-cold Hanks' balanced salt solution containing 25 mM HEPES (HHBSS) and 12.5 units/mL penicillin, 6.5 μg/mL strepomycin, and 5% bovine serum albumin. Nonspecific binding was assessed by incubating some of the wells in HHBSS without Ca^{2+} or Mg^{2+} containing a 100-fold excess of either unlabeled α_2M^* or RBF. Increasing concentrations of ^{125}I-ligands (α_2M^* or RBF) were added to the wells which were then incubated for 16–18 h at 4 °C as previously described (Howard et al., 1996a,b). When the effect of Ni^{2+} was

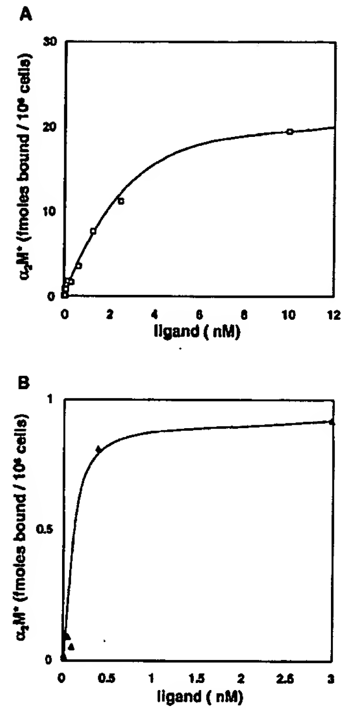

FIGURE 2: The effect of Ni^{2+} on the binding of α_2M^* to macrophage LRP/α_2MR and α_2MSR. [^{125}I]α_2M^* specific binding as a function of ligand concentration was examined in the presence of Ni^{2+} at a fixed concentration, 5 mM. Panel A shows the binding isotherm in the absence of Ni^{2+} and panel B in the presence of Ni^{2+}. The *y*-axis in panel B has been greatly expanded in comparison to panel A so that the specific binding in the presence of Ni^{2+} can be discerned. Details of the binding assay are contained in the Materials and Methods.

studied, the metal ion was present during the entire incubation period. Concentrations of Ni^{2+} between 1 and 10 mM were studied. All binding studies were performed at least three to five times and in triplicate. Standard errors of the mean for these studies were ≤10%. Scatchard analysis was used to analyze the binding data as previously reported (Howard et al., 1996a,b).

In one series of studies, the specific binding of α_2M^* (0.5 nM) was studied at increasing concentrations of Ni^{2+}. These studies were performed at 4 °C for cells which were incubated with or without Ni^{2+} for 16–18 h. The study was performed on five occasions in triplicate. Standard errors of the mean for these studies were ≤10%.

IP_3 and Ca^{2+} Measurements. IP_3 was quantified as described by Berridge (1983) and previously reported in detail (Misra et al., 1993, 1994a,b; Howard et al., 1996a,b). In brief, macrophage monolayers were incubated with 2-[^{3}H]-*myo*-inositol (8 μCi/mL) in each well at 37 °C for 16–18 h. Monolayers were then rinsed three times in HHBSS containing 1 MCaCl$_2$, 1 mM MgCl$_2$, 10 mM LiCl, pH 7.4. The cells were then incubated in this medium with ligand for various time periods. The reaction was stopped by removing the medium and adding 6.25% perchloric acid solution. The cells were placed in tubes containing 1 mL of octylamine/Freon (1:1 v/v) containing 5 mM EDTA. The tubes were then centrifuged at 5600*g* for 20 min at 4 °C. The upper phase was applied to a 1 mL column of AG1-X8 formate Dowex resin (Bio-Rad Laboratories, Richmond, CA). The column was eluted serially with batches of ammonium formate solution containing 0.1 M formic acid as described

(Misra et al., 1993). Aliquots were then counted by liquid scintillation. For studies of the effect of Ni^{2+}, the metal ion was present at 5 mM for the time period of incubation of the cells with α_2M^*.

For measurements of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), marophages were plated on glass coverslips placed in 35 mm Petri dishes at a density of 1.5×10^5 cells/cm^2 and incubated in RPMI medium for 16−18 h in a humidified incubator at 37 °C with 5% CO_2. The cells were then treated with Fura-2/AM (1−1.5 μM) containing medium at room temperature for 30 min in the dark. The monolayers were then washed twice in HHBSS containing 75 μM Ca^{2+}. Under these conditions, macrophage capacitative entry of Ca^{2+} is minimal when the cells are exposed to α_2M^* (Misra et al., 1993). These conditions were employed to allow the effect of Ni^{2+} to be studied. Ni^{2+} is capable of blocking uptake of Ca^{2+} from the extracellular medium; however, this cation does not penetrate the cells and does not affect the mobilization of Ca^{2+} from intracellular pools (Kwan & Putney, 1990). For studies of the effect of Ni^{2+}, the cation was present at a concentration of 5 mM during the incubation of cells with α_2M^*.

Measurements of $[Ca^{2+}]_i$ were obtained employing a digital imaging microscope as described in detail elsewhere (Misra et al., 1993). A baseline measurement in buffer was obtained for the macrophage monolayers at 37 °C before adding α_2M^*. When Ni^{2+} (5 mM) was employed in studies, it was present in the buffer prior to addition of the α_2M^*. $[Ca^{2+}]_i$ was then monitored at 37 °C. Ni^{2+} at this concentration had no effect on trypan blue exclusion compared to control cells similarly treated for periods of at least 15 min at 37 °C, a time sufficient for completion of IP_3 and $[Ca^{2+}]_i$ measurements. This observation is in accord with previous studies (Kwan & Putney, 1990).

RESULTS

Effect of Ni^{2+} on the Cellular Binding of α_2M^*. Consistent with previous observations employing purified LRP/α_2MR or smooth muscle cells (Hussain et al., 1995), Ni^{2+} blocks the binding of [^{125}I]α_2M^* (0.5 nM) to murine peritoneal macrophages (Figure 1). In the present studies, the maximal effect of Ni^{2+} was achieved at a $[Ni^{2+}]$ of about 5 mM. This concentration was employed in the subsequent binding studies. The specific binding of [^{125}I]α_2M^* never approached zero in any of our studies.

The binding of [^{125}I]α_2M^* as a function of concentration was then studied in the absence or presence of Ni^{2+} (Figure 2). The binding of [^{125}I]α_2M^* was suppressed to extremely low levels at all concentrations of [^{125}I]α_2M^* studied in the presence of Ni^{2+} (5 mM) (Figure 2). In order to analyze the data in greater detail, Scatchard analysis was then employed. As can be seen, the effect of Ni^{2+} was restricted to the lower affinity site ($K_d \approx 10$ nM) (Figure 3) previously identified as LRP/α_2MR (Howard et al., 1996a,b,d). Binding to the very high affinity α_2M^* binding site ($K_d \approx 50$ pM) was unaffected. This class of binding sites has previously been identified as α_2MSR (Howard et al., 1996a−c). This effect is best seen by comparing the effect of two Ni^{2+} concentrations, 1 and 5 mM on the two classes of binding sites (Figure 3, panels B and C). Only the lower affinity class of sites shows dose-dependent suppression of the binding of [^{125}I]α_2M^* (Figure 3, panels B and C).

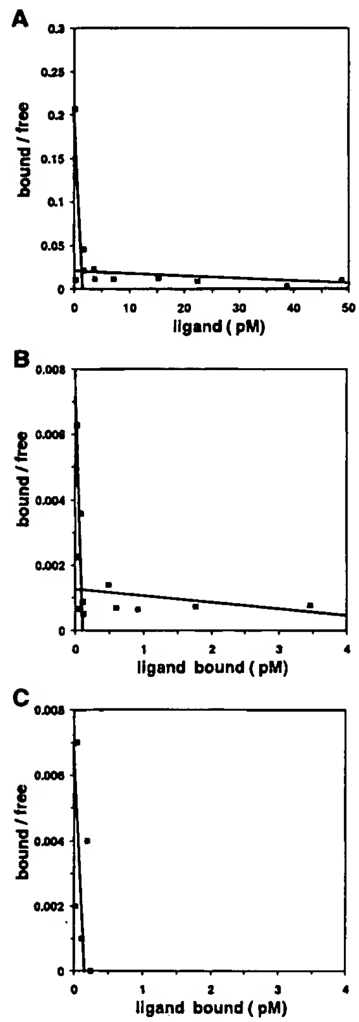

FIGURE 3: Scatchard analysis of the binding of α_2M^* in the presence of Ni^{2+} to macrophage LRP/α_2MR and α_2MSR. The Scatchard replots are for the data in Figure 2 as well as for a study in the presence of 1 mM Ni^{2+}. Panel A represents the data obtained in the absence of Ni^{2+}, panels B and C in the presence of a $[Ni^{2+}]$ of 1 and 5 mM, respectively. The y-axis in panels B and C have been significantly expanded to better discern the effect of Ni^{2+} on the binding of [^{125}I]α_2M^* to LRP/α_2MR (lower affinity site). Details of the binding assay are contained in the Materials and Methods.

Effect of Ni^{2+} on the Cellular Binding of RBF. The effect of Ni^{2+} on the binding of RBF to LRP/α_2MR and α_2MSR was next investigated (Figure 4). In contrast to the observations made when α_2M^* was the ligand, Ni^{2+} had no effect on the binding of [^{125}I]RBF to LRP/α_2MR. [^{125}I]RBF binding to α_2MSR was also unaffected by Ni^{2+}. The observations made with respect to the binding of RBF to LRP/α_2MR suggests that a portion of the LRP/α_2MR receptor binding site is present in a region of α_2M upstream of RBF. This observation is consistent with a significant number of previous observations from this laboratory suggesting a similar conclusion (Pizzo et al., 1986; Enghild et al., 1989; Isaacs et al., 1988; Roche et al., 1988; Howard et al., 1996a).

Effect of Ni^{2+} on IP_3 Synthesis and $[Ca^{2+}]_i$. The effect of Ni^{2+} on the ability of α_2M^* to induce IP_3 synthesis by ligation of macrophage α_2MSR was then studied (Figure 5). At a $[Ni^{2+}]$ of 5 mM there was no decrease in IP_3 synthesis induced by α_2M^* (100 pM). The shape of the curve was, however, somewhat different with a higher peak value and prolonged increase in IP_3 synthesis above the basal level.

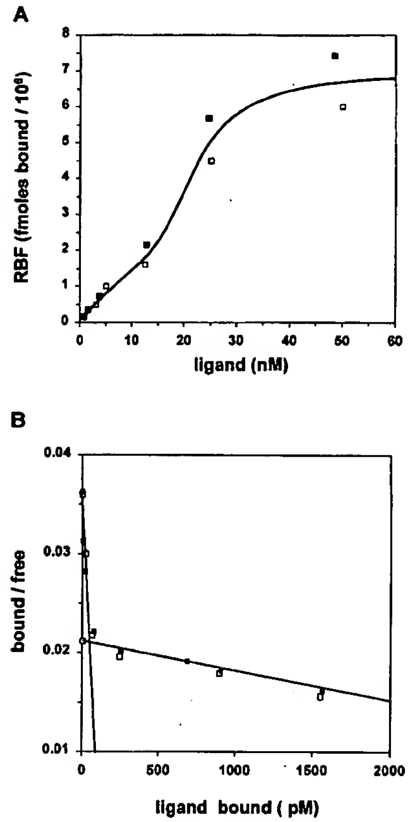

FIGURE 4: The effect of Ni^{2+} on binding of RBF to macrophage LRP/α_2MR and α_2MSR. Panel A shows the binding of [^{125}I]RBF macrophages in the presence of Ni^{2+} at a fixed concentration of 5 mM (■). The binding curve was essentially identical to that obtained in the absence of Ni^{2+} (□). Panel B shows the Scatchard replot of the data from panel A. Details of the binding assay are contained in the Materials and Methods.

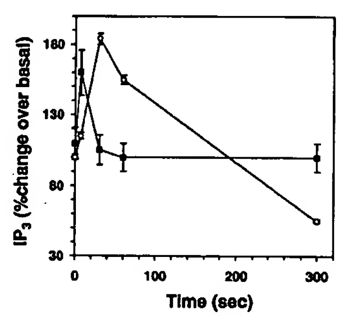

FIGURE 5: The effect of Ni^{2+} on IP_3 synthesis induced in macrophages by α_2M*. Macrophages were exposed to α_2M* (100 pM) in the absence (■) or presence of (O) a fixed concentration of Ni^{2+} (5 mM). An IP_3 dose-response curve with α_2M* in the range 0−60 pM was also obtained in the presence and absence of Ni^{2+} at a fixed concentration of 5 mM. These curves were identical and are not shown. The values reported are from three separate experiments performed in triplicate. The values are shown as the mean ± the standard error of the mean.

Ligation of α_2MSR by α_2M* or RBF causes a 2−3-fold IP_3-dependent rise in macrophage $[Ca^{2+}]_i$ (Misra et al., 1993, 1994a,b). The ability of α_2M* (100 pM) to induce an increase in macrophage $[Ca^{2+}]_i$ was, therefore, examined in the presence or absence of Ni^{2+} (5 mM). The absolute rise in $[Ca^{2+}]_i$ was comparable and greater than 2-fold in each case (Table 1). While the absolute increase in $[Ca^{2+}]_i$ was comparable in the presence and absence of Ni^{2+}, we also observed a more prolonged response to α_2M* in the presence of Ni^{2+}. This is difficult to quantify or represent since it is based on the visualization of many individual cells. However, visualizing at least 100 cells for each experimental group suggests that the duration of the increase in $[Ca^{2+}]_i$ induced by α_2M* in the presence of Ni^{2+} is 2−3-fold greater in duration than the increase induced by α_2M* alone.

Table 1: Effect of Ni^{2+} on the Ability of α_2M* to Stimulate Increases in $[Ca^{2+}]_i$ in Macrophages

treatment[a]	basal	ligand-induced	fold increase
α_2M*	153.7 ± 11.3	347.9 ± 22.1	2.26
α_2M* + Ni^{2+}	117.0 ± 4.9	262.8 ± 33.6	2.25

[a] Macrophages were treated with α_2M* (100 pM) in the absence or presence of Ni^{2+} (5 mM). The peak increase in $[Ca^{2+}]_i$ is indicated for cells exposed to ligand and the baseline before ligand addition is indicated as the basal level. The stimulated values represent the peak response seen between 1 and 2 min after the cells were exposed to α_2M* with or without Ni^{2+}. At least 100 cells were studied on several different days for both experimental conditions. The values reported are the mean ± the standard error of the mean. The results are comparable to previous data reported from our laboratory (Misra et al., 1993; 1994a,b).

The effect of Ni^{2+} (5 mM) on the ability of RBF to induce both macrophage IP_3 synthesis and increases in $[Ca^{2+}]_i$ was also studied. As with α_2M*, Ni^{2+} had no effect on either IP_3 synthesis or $[Ca^{2+}]_i$ induced by RBF (data not shown).

DISCUSSION

Two distinct receptors bind receptor-recognized forms of α_2M; namely, LRP/α_2MR and α_2MSR. Binding of α_2M* to LRP/α_2MR is clearly an important catabolic pathway for uptake of α_2M−proteinase complexes (Salvesen & Pizzo, 1993; Kriger & Herz, 1994; Chu & Pizzo, 1994). It has been estimated that during a single day humans turn over as much as a gram of α_2M by virtue of proteinase activation and uptake of the complexes by the liver and other organs (Salvesen & Pizzo, 1993). The role of α_2MSR is less clearly understood since this receptor was only recently identified (Misra et al., 1994a,b). Ligation of this receptor regulates signal transduction cascades in a variety of cells. These events are linked to DNA synthesis and cellular proliferation of both smooth muscle and rheumatoid synovial cells (Webb et al., 1995; Misra et al., 1997). These effects are seen at extremely low concentrations of ligand, reflecting the very high affinity of α_2M* or RBF for α_2MSR ($K_d \approx 50$ pM).

In the present study, we demonstrate that Ni^{2+} blocks the binding of α_2M* to LRP/α_2MR but not α_2MSR. Previous investigators have demonstrated that Ni^{2+} blocks the binding of α_2M* to LRP/α_2MR (Hussain et al., 1996); however, these investigators did not examine the effect of Ni^{2+} on α_2MSR or signal transduction. While Ni^{2+} did not affect the binding of α_2M* to α_2MSR, we did find some effect of Ni^{2+} on the ability of α_2M* to promote IP_3 synthesis and increases in $[Ca^{2+}]_i$. This effect took the form of a prolonged increase in both IP_3 synthesis and the rise in $[Ca^{2+}]_i$ induced by α_2M* in the presence of Ni^{2+}. The reason for this effect is unclear. In part, it may reflect the fact that, by blocking LRP/α_2MR-mediated uptake of α_2M*, receptor occupancy of α_2MSR may be prolonged. By analogy, it has been shown that LRP/α_2MR, by virtue of its ability to bind urinary-type plasminogen activator (u-PA), also affects receptor occupancy of the u-PA receptor, UPAR. Ligation of UPAR promotes DNA synthesis and cellular proliferation by activation of a signaling cascade (del Rosso et al., 1993; Dumler et al.,

1993). This receptor is not capable of promoting ligand uptake which appears to require the coexpression of LRP/α_2MR on cells (Grobmyer et al., 1993). LRP/α_2MR is capable of binding and promoting uptake of the u-PA/UPAR complex thus terminating a signaling event. This appears to be the primary mechanism for regulating the growth factor activity of u-PA.

In contrast to the effect of Ni^{2+} on α_2M^*, Ni^{2+} had no effect on the binding of RBF to LRP/α_2MR. The effect, therefore, of Ni^{2+} on α_2M^* may involve a region of the protein upstream from the carboxy terminal receptor recognition domain. This observation is of interest in view of previous studies from this laboratory with regard to the effects of *cis*-dichlorodiamineplatinum(II) (*cis*-DDP) on the receptor recognition of α_2M^*. *cis*-DDP affects amino acid residues which are not in the carboxyl terminal receptor recognition domain (Roche et al., 1988; Enghild et al., 1989; Howard et al., 1996a), but in a region which is upstream of the receptor binding domain (Enghild et al., 1989). While RBF does incorporate a small amount of *cis*-DDP (Enghild et al., 1989), this reaction also has no effect on the binding of RBF to α_2MSR (Howard et al., 1996a). Similarly oxidation of RBF does not alter its interaction with α_2MSR (Wu & Pizzo, 1997). It is of interest that exposure of RBF to Ni^{2+} also has no effect on its interaction with α_2MSR.

The effect of Ni^{2+} on the binding of ligands to LRP/α_2-MR appears restricted to α_2M^* since Hussain et al. (1995) found no effect of Ni^{2+} on the binding of *Pseudomonas* exotoxin A, LDL, or RAP to this receptor. We have confirmed these observations and also have extended them to lipoprotein lipase and lactoferrin with similar results (U. K. Misra, and S. V. Pizzo, unpublished observations). Hussain et al. (1995) demonstrated that LRP/α_2MR bound Ni^{2+} and they concluded that the Ni^{2+} effect was the result of binding this cation to the receptor. Our data obtained with RBF suggest that this hypothesis may need to be reconsidered. Clearly, RBF and α_2M^* bind to the same site on LRP/α_2MR (Enghild et al., 1989; Howard et al., 1996a–d). The lack of a Ni^{2+} effect on RBF binding to LRP/α_2-MR may suggest that binding of Ni^{2+} to α_2M^*, rather than LRP/α_2MR, accounts for the loss of binding of α_2M^* to LRP/α_2MR in the presence of Ni^{2+}.

The lack of an effect of Ni^{2+} on LDL binding to LRP/α_2MR may also be inconsistent with the original hypothesis. We have recently demonstrated that LDL and α_2M^* bind to the same domain on LRP/α_2MR (Wu & Pizzo, 1996). This is unusual in that with the exception of RAP, few ligands cross-compete for binding to LRP/α_2MR (Krieger & Herz, 1994). Since LDL and α_2M^* both bind to the same domain on LRP/α_2MR (Wu & Pizzo, 1996), the reason for the differential effect of Ni^{2+} on the binding of these two ligands to this receptor is unclear assuming that the Ni^{2+} effect is dependent on the binding of this metal ion to LRP/α_2MR. The study of Hussain et al. (1995) did show binding of Ni^{2+} to α_2M^* as well as to LRP/α_2MR. Moreover, we have previously employed Ni^{2+} chelate chromatography to purify a number of α-macroglobulins including bovine, human, chicken, and frog (Feldman et al., 1984; Feldman & Pizzo, 1985, 1989a,b). Whether this binding accounts for the differential effect of Ni^{2+} on RBF and α_2M^* binding to LRP/α_2MR will require further investigation.

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BI970806K

1: Mol Med Today. 1998 Nov;4(11):478-84. Related Articles, Links

Heat shock protein--peptide complexes as immunotherapy for human cancer.

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Heat shock proteins (Hsps), ubiquitous in nature, act as chaperones for peptides and other proteins. They have been implicated in loading immunogenic peptides onto major histocompatibility complex molecules for presentation to T cells. When isolated from tumor cells, Hsps are complexed with a wide array of peptides, some of which serve as tumor-specific antigens. Animal studies have demonstrated that heat shock protein--peptide complexes (HSPPCs) from tumor cells can act as vaccines to prevent or treat tumors. Potent and specific tumor antigens have long been the holy grail in cancer immunotherapy; HSPPCs from tumor cells could become a safe and reliable source of tumor-specific antigens for clinical application.

Publication Types:
- Review
- Review, Tutorial

PMID: 9857367 [PubMed - indexed for MEDLINE]

[CANCER RESEARCH 48, 6715–6720, December 1, 1988]

Investigation of the Effects of Heat Shock and Agents Which Induce a Heat Shock Response on the Induction of Differentiation of HL-60 Cells[1]

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ABSTRACT

A heat shock of 42.5–43.5°C for 1 h applied to HL-60 promyelocytic leukemia cells induced the appearance of between 13 and 34% ($n = 6$) of cells which showed characteristics of mature metamyelocytes/granulocytes. This is the first time a physical agent has been shown to induce the differentiation of this leukemic cell line. The treatment of HL-60 cells with a variety of agents which have been documented to stress cells and induce thermotolerance or a heat shock-like response also induced granulocyte-like differentiation: continuous treatment for 4 days with ethanol (213 mM), sodium arsenite (6 μM), cadmium sulfate (60 μM), lidocaine (3 mM), and procaine (5 mM) induced 73, 54, 14, 54, and 55% of cells, respectively, to reduce the dye nitro blue tetrazolium. They were also capable of the phagocytosis of yeast particles. Examination of differentiated cells showed that those treated with ethanol, arsenite, lidocaine, and procaine also expressed nonspecific esterase activity, typical of monocytes, but did not adhere to plastic and had a cellular and nuclear morphology consistent with differentiation to metamyelocytes. Analysis of protein synthesis of HL-60 cells treated with 170 mM *N*-methylformamide, by the pulse labeling of cells for 2 h with [^{14}C]leucine at various times, showed that the constitutive synthesis of both the M_r 90,000 and 70,000 heat shock proteins fell substantially after 2 h of exposure to *N*-methylformamide. When HL-60 cells were incubated with 1 M *N*-methylformamide, a toxic concentration of this agent, or were heat shocked, the synthesis of both the M_r 70,000 and M_r 90,000 proteins was induced. We propose that changes in heat shock protein synthesis may be an important element of the induction of differentiation of HL-60 cells, particularly as these proteins have recently been shown to regulate the stability of oncogene proteins, such as *myc* (Lüscher, B., and Eisenman, R. N., Mol. Cell Biol., *8:* 2504–2512, 1988).

INTRODUCTION

A wide variety of drugs and toxins are capable of the promotion of the terminal differentiation of HL-60 human promyelocytic leukemia cells to granulocyte-like cells. These include the polar solvents (2, 3), anthracyclines (4–6), antimetabolites (7–9), an inhibitor of glycoprotein synthesis (10), retinoids (11–12), and benzodiazepines (13). These disparate agents are optimally active at concentrations which are only marginally below those which are cytotoxic, and in the case of the solvents, and related compounds, a quantitative relationship was defined by us which related the concentrations required for toxicity to those required for the induction of differentiation (3). We proposed that the treatment of cells with agents at subtoxic concentrations may serve to "stress" the cells and induce some kind of adaptive or "stress" response (3). It was considered possible that such a response may be involved in those events which result in changes of gene expression leading to terminal cell differentiation, including changes in the expression of certain oncogenes, the activity of which may be responsible for the failure of these cells to mature and for their malignancy.

Recently, there have been suggestions that a family of proteins, which are evolutionarily highly conserved, the hsps,[4] may play an important role in the control of cellular differentiation (14–21). The hsps belong to a class of proteins which are induced in all eukaryotic cells in response to an imposed stress (reviewed in Reference 14), such as a shift to a higher temperature ("heat shock") or other agents, for example ethanol and other polar solvents (22–24). It is considered that the modification of proteins may be an important trigger in the initiation of the heat shock response (25–27). In addition, certain of the hsps have been shown to modify the degradation of short-lived oncogene proteins such as p53 (28, 29) and *myc* (30) and may thus play a role in malignancy.

In order to test the hypothesis that the imposition of a relatively nonspecific stress may bring about HL-60 cell differentiation, we have investigated the effects of a heat shock, and of agents which have been reported to induce the synthesis of hsps, on the growth and differentiated phenotype of HL-60 cells. We also present data on hsp synthesis during the period that HL-60 cells are committed to differentiation by the agent *N*-methylformamide.

MATERIALS AND METHODS

Cell Culture. HL-60 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO, Glasgow) at 37°C, in an atmosphere of 10% CO_2 in air. They were maintained in logarithmic phase of cell growth by serial subculture of between 5×10^4 and 1×10^6 cells/ml at 3-day intervals. A doubling time of between 20 and 24 h was recorded. Initiation of cell growth at 1×10^5 cells/ml allowed a plateau phase of growth to be reached when the cell density was between 1 and 2×10^6 cells/ml. Cell numbers were estimated by the use of a Coulter counter (model ZB_1) and short-term viability was determined by the exclusion of a 0.1% solution of trypan blue.

Induction of Differentiation. (*a*) By heat shock: 1×10^6 HL-60 cells/ml were exposed to an immediate heat shock (*i.e.*, no preheating) of between 39 and 45°C for various times in a thermostatted water bath, the temperature of which was regulated by a Mercia Scientific (Coventry, UK) thermostat to within ±0.5°C. Measurements of the rate of heating of the culture showed that with adequate agitation the media reached the required temperature within 2 min. At the end of the required period of heat shock, cells were allowed to stand at 21°C for 5 min, by which time they had cooled to ambient temperature, then incubated at 37°C for a further 96 h; cell viability, cell number, incorporation of radiolabeled thymidine and % differentiation (see below) were assayed immediately and at various times. (*b*) By other agents: 1×10^5 HL-60 cells/ml were incubated continuously for 96 h with various concentrations of the agents, and cell number, % viability, and % differentiation assessed as described below.

Assessment of Differentiation. HL-60 cells which were potentially capable of the production of superoxide anion were assessed by the reduction of the dye nitrobluetetrazolium, as described in detail by us previously (3). Cells which were functionally capable of phagocytosis were assessed by the method of Shaala *et al.* (31). Cytochemical demonstration of nonspecific esterase activity was performed according to

Received 1/20/88; revised 8/2/88; accepted 9/1/88.

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[1] Supported by Grant SP1518 from the Cancer Research Campaign. Parts of this work have been reported elsewhere in abstract form (1).

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[4] The abbreviations used are: hsp(s), heat shock proteins; DMSO, dimethylsulfoxide; NBT, nitro blue tetrazolium; NMF, *N*-methylformamide.

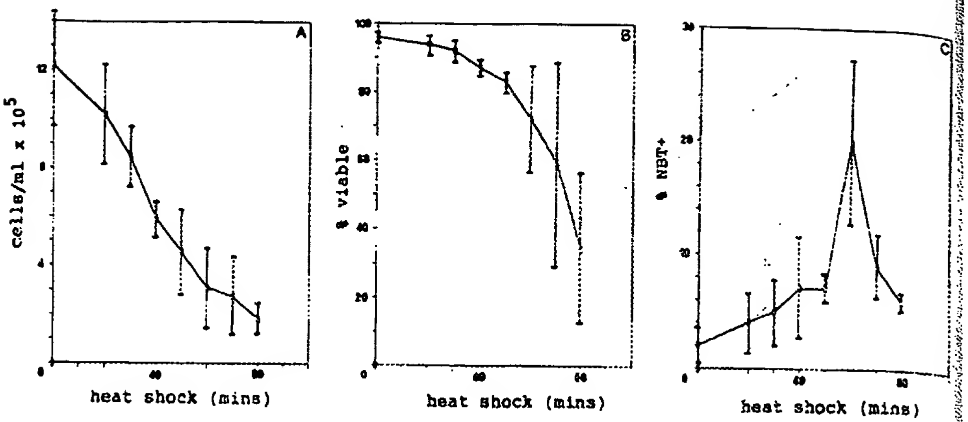

Fig. 1. Induction of differentiation of HL-60 cells by a 43.5°C heat shock. *A*, effect of the time of exposure to the heat shock on cell density, measured after 4 days of incubation at 37°C. *B*, effect of various times of exposure to the heat shock on cell viability, measured by the exclusion of trypan blue after 4 days of incubation at 37°C. *C*, percentage differentiation of heat shocked HL-60 cells, measured as the percentage which reduced the dye NBT after 4 days of incubation at 37°C. (The mean values of six experiments).

the method of Yam *et al.* (32). Microscopical assessment of the morphology of the cells was made after they had been stained with Wolbach-Giemsa stain (33). DNA synthesis was measured by incubation of 10^6 cells with 0.5 μCi of [5-*methyl*-^{3}H]thymidine (5 Ci/mmol; Amersham, UK) for 0.5 h before placing them, in triplicate, on a 2.5-cm Whatman GF/C glass fiber filter, washing with 15 ml each of normal saline, 10% w/v trichloroacetic acid, and a further saline wash. The filters were washed with 15 ml of methanol then air dried, and the radioactivity of each filter was measured in a Packard 2000 CA Tricarb scintillation counter after the addition of Optiphase (Fisons, Loughborough, UK). Preliminary experiments had shown incorporation of the thymidine into acid-insoluble material in the cells was linear for at least 1 h.

Measurement of New Protein Synthesis. Cells were untreated or heat shocked for 1 h at 43.5°C and allowed to recover for 2 h at 37°C, or were treated with various concentrations of NMF or ethanol for different times. The cells were washed twice with leucine-free RPMI 1640, then 5×10^6 cells were incubated for 2 h at 37°C with 2 ml of leucine-free RPMI plus 10% dialyzed fetal calf serum and 3 μCi of L-[U-^{14}C]-leucine (342 Ci/mmol). The samples were washed twice with leucine-free RPMI, then twice with reticulocyte standard buffer (0.01 M Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$) before resuspending in 1 ml of buffer. The cells were sonicated, then sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (34). Electrophoretic transfer of the proteins onto 0.45-μm nitrocellulose (Biorad, Watford, UK) was then performed according to the method of Towbin (35). The dried blot was placed on a (CEA) Singul X-RP nonscreen medical X-ray film (Ceaverken AB, Strangas, Sweden), and exposed in the dark for 3 days before the film was developed. Scanning densitometry was performed using an LKB Ultrascan XL laser densitometer.

RESULTS

A heat shock for 1 h between 42.5 and 43.5°C resulted in the maintenance of a short-term viability of >85% of HL-60 cells as assessed by the exclusion of trypan blue at the end of the heat shock period. Experiments aimed at the assessment of whether heat-shocked cells would subsequently differentiate and divide were conducted at these temperatures. Fig. 1 shows the effect of a heat shock of 43.5°C applied for time periods of up to 80 min, on the viability, cell number, and percentage of cells capable of reducing the dye NBT (termed NBT+ cells), assessed 96 h after return of the cells to 37°C. It shows the averaged results from a series of six experiments; the highest percentage of NBT+ cells after a 1-h heat shock at this temperature was 34% and the lowest 13%. At time intervals before 1 h, less than 5% differentiation was observed, and at longer periods than 1 h cell viability began to decline, with no increase in the numbers of NBT+ cells (Fig. 1). Estimates of cell numbers after 96 h showed that after a 1-h heat shock those cells which had differentiated had gone through just over one cell division, from 1×10^5 cells to $3.1 \pm 1.0 \times 10^5$ cells/ml, whereas the control cells had completed over three divisions. Heat shock for up to 4 days at temperatures of below 42°C did not result in significant differentiation (data not shown). It was established that the cells did not express nonspecific esterase activity typical of monocytic cells, but were capable of the phagocytosis of complement-coated yeast particles (see below).

In a further series of heat shock experiments, the rate of development of the differentiated phenotype and changes in DNA synthesis were monitored over a 72-h period immediately following a 1-h heat shock at 42.5°C. Short term viability of the heat-shocked cells, measured by the exclusion of the vital dye trypan blue, remained at >80% over 72 h. Surprisingly, heat-shocked cells rapidly expressed the capability of the phagocytosis of yeast particles and were able to reduce the dye NBT (Fig. 2). We attempted to establish whether there was a significant fall in DNA synthesis, as measured by [^{3}H]thymidine incorporation, as the cells recovered from heat shock and began to express markers representative of a more mature phenotype. No significant depression of thymidine incorporation was observed in the heated cells over a 72-h period following the heat shock. We consider that it is not possible to confirm, using thymidine incorporation, whether the approximately 25% of cells which expressed markers of a more mature phenotype were terminally differentiated because of the superimposition of the growth of the 75% of cells which remained uncommitted, and the possibility that the heat-shocked cells were capable of performing nonscheduled DNA synthesis after receiving the heat shock.

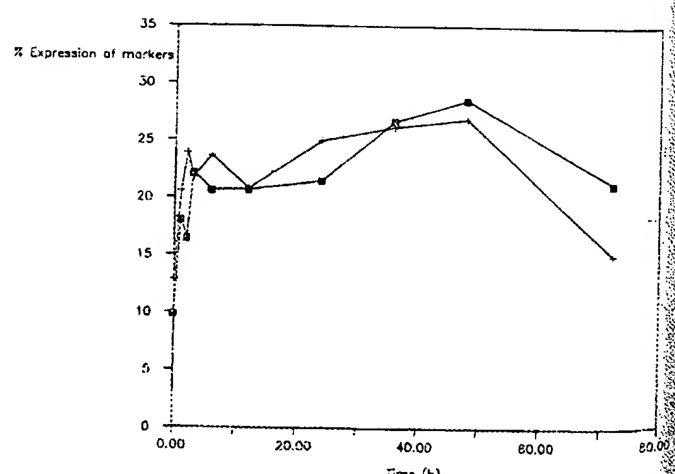

Fig. 2. The time course of the percentage expression of markers of a phenotype typical of metamyelocytes/granulocytes after exposure of HL-60 cells to a heat shock of 42.5°C for 1 h. +, phagocytosis of yeast particles; ■, reduction of NBT. (The data shows the mean values from two experiments.)

Table 1 *Effects of inducers of the heat-shock response on cell growth and differentiation of HL-60 cells measured after 96 h (±SD)*

Treatment	Cell number × 10^5 after 96 h[a]	% Viable[b]	% NBT+	% Phagocytosis	% NSE	N
None	12 ± 0.3	96 ± 3	2 ± 1	1 ± 1	<1	6
170 mM NMF	2.6 ± 0.3	86 ± 9	83 ± 10	76 ± 12	<1	6
213 mM ethanol	2.3 ± 0.5	82 ± 13	73 ± 11	69 ± 11	22 ± 1	3
6 μM Na arsenite	2.6 ± 0.4	80 ± 6	54 ± 7	51 ± 10	37 ± 16	3
3 mM lidocaine HCl	2.8 ± 1.2	83 ± 8	54 ± 2	52 ± 3	45 ± 5	3
5 mM procaine HCl	2.5 ± 0.9	84 ± 7	55 ± 4	54 ± 3	49 ± 4	3
60 μM cadmium sulfate	3.5 ± 1.6	75 ± 16	14 ± 11	Not done	Not done	6
1 h at 43.5°	3.1 ± 1	72 ± 13	20 ± 7	23 ± 2	<1	6

[a] Initial cell number was 1 × 10^5 cells/ml.
[b] Short term viability as measured by dye exclusion, see "Materials and Methods."

Table 1 shows the effects on growth and differentiation of HL-60 cells after treatment for 96 h with a variety of agents which have been documented to induce thermotolerance and/or the expression of hsps (22–24, 36–38). The optimal concentration for the induction of differentiation is as defined previously by us (3). That is, that concentration which reduces cell division and induces the expression of the maximum number of NBT+ cells, while viability was maintained at approximately 80%. Ethanol, procaine, lidocaine, and sodium arsenite are what have been defined as "strong" inducers (39), while cadmium appears to be only a "partial" inducer, and like a heat shock its effects were rather more variable than the other agents (Table 1). We attempted to assay the activity of a variety of lead salts but found all of them to precipitate in the medium, no doubt because of their reaction with sulfhydryl-containing components.

Table 1 also shows that cells which had been treated with some of these agents gave rise to a proportion of cells which expressed significant levels of nonspecific esterase activity more typical of the monocytic pathway of differentiation. The cultures however, did not display any cell attachment to the surface of the falcon flasks. In addition, Giemsa staining and examination of the nuclear morphology of the cells clearly showed reniform and occasionally banded nuclei, typical of metamyelocytes of the neutrophil-granulocyte lineage (40). The plasticity of the leukemic genome has been commented on by Greaves *et al.* (41); the data presented here support these views, and additionally suggest that it is useful to measure a number of markers of differentiation.

Fig. 3 shows the autoradiograph of the nitrocellulose blot of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the new proteins synthesized, in an equal number of cells, after the cells had been pulsed for 2 h with [^{14}C]leucine. Prior to labeling with leucine the cells were either untreated, heat shocked for 1 h at 43.5°C and allowed to recover at 37°C for 2 h, or treated with NMF or ethanol for various times. The blot is presented as the autoradiograph of this is slightly better resolved than that of the original gel. Clearly, those cells which had been subjected to the heat shock (*Lane J*) ceased the synthesis of many proteins. The most prominent new proteins synthesized in the heat-shocked cells were those of the major hsp families of M_r 70,000, 90,000, and 110,000. Treatment of the cells with a toxic concentration (3) of NMF (1 M) (*Lane H*) or ethanol (1.28 M) (*Lane I*) for 1 h also induced the synthesis of the major hsps; these agents had less effect on normal protein synthesis than a heat shock.

Fig. 3 (*Lanes A* to *G*) also shows the effects, with time, on protein synthesis after HL-60 cells had been incubated with a concentration of NMF which was optimal for the induction of differentiation (Table 1 and Reference 3). In the untreated cells (*Lane A*) there appears to be considerable constitutive synthesis of the M_r 90,000 hsp (which appears to be a doublet), and some constitutive synthesis of the M_r 70,000 hsp. Incubation with 170 mM NMF rapidly reduced the constitutive synthesis of these proteins and Fig. 4 shows the results of a densitometric scan of the gel comparing the absorbance of the M_r 90,000 peak (both bands of the poorly resolved doublet) with that of the M_r 45,000 peak, which corresponds to actin. The synthesis of actin had a densitometric average absorbance of 0.36 ± 0.035 units ($n = 6$).

DISCUSSION

We have tested the hypothesis that a number of disparate agents which have been reported to "stress" cells, possibly by damaging proteins, and which are documented to induce the expression of hsps, might promote the differentiation of HL-60 cells. The finding that a heat shock could induce HL-60 cells to express markers typical of granulocyte-like cells, with the capability of superoxide production under conditions of reduced cell proliferation, is a novel observation (Fig. 1) since it is the first physical agent recorded to promote the differentiation of these cells. Although a significant but rather low degree of differentiation was induced by heat shock, this was variable to a degree not normally seen when chemical agents are used. The reason for this variability is unclear at present. The experiments which measured the induction of hsps in these cells (Fig. 3, *Lane J*) show that a heat shock had a profound inhibitory effect upon normal protein synthesis while it induced the synthesis of the major hsps: it is possible therefore that the expression of a differentiated phenotype was inhibited by these conditions (cadmium had a similar effect on protein synthesis[5]). However, ethanol, which as discussed below elicits many of the stress responses induced by heat but apparently, unlike heat, does act as a general inhibitor of protein synthesis (42), was a consistently strong inducer of differentiation (Table 1), and treatment with even a toxic concentration of this agent (Fig. 3, *Lane I*) had a less profound inhibitory effect on general protein synthesis in HL-60 cells than did heat shock. Similarly, a toxic concentration of NMF (*Lane H*) had little effect on normal protein synthesis, while it induced the synthesis of the major heat shock proteins. We considered that the imposition of a stress by heat shock was too variable a procedure for detailed analysis of the accompanying cellular and biochemical events and turned to other agents, as discussed below.

A feature of note regarding the promotion of differentiation by a heat shock was that significant differentiation, up to 34% in one experiment, was induced after only a 1-h exposure to this physical agent. Experiments to measure the minimum time of exposure to the polar solvent NMF, which was required to induce significant levels of differentiation, suggest that a minimum of 24 h was necessary,[5] while not less than 8 h of

[5] F. M. Richards and J. A. Hickman, unpublished.

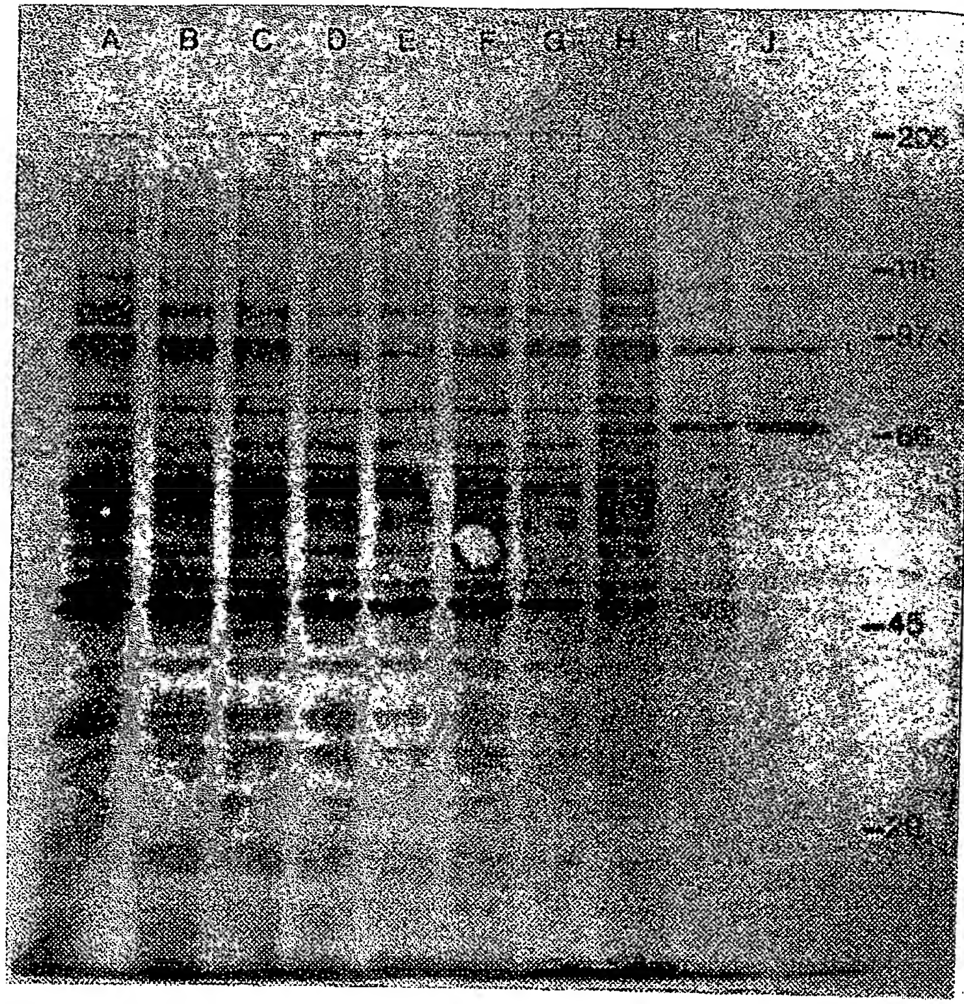

Fig. 3. Autoradiograph of the nitrocellulose blot of an SDS-PAGE to show protein synthesis in *A*: nontreated HL-60 cells; in cells incubated continuously with 170 mM NMF for: *B*, 2 h; *C*, 6 h; *D*, 20 h; *E*, 30 h; *F*, 44 h; *G*, 54 h; or for 1 h, followed by 2-h recovery, with *H*, 1 M NMF; *I*, 1.3 M ethanol; *J*, a 1-h heat shock for 43.5°C.

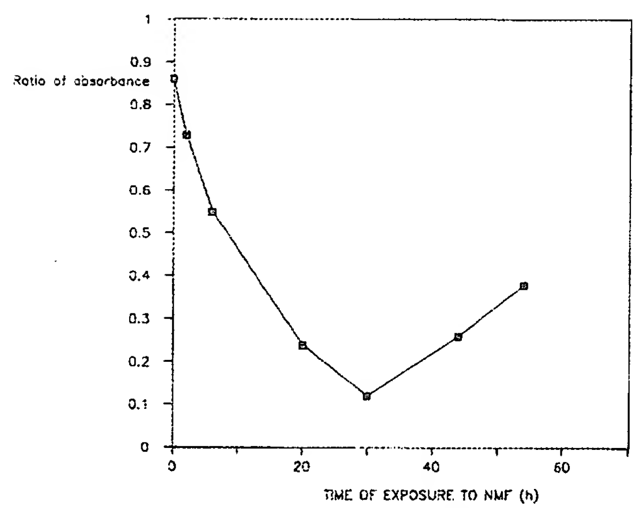

Fig. 4. Densitometry of the autoradiograph shown in Fig. 3, in which the absorbance of the peak corresponding to the M_r 90,000 protein was compared with that of the peak at M_r 45,000, corresponding to actin, over the 56-h time period of incubation of HL-60 cells with 170 mM NMF.

incubation with DMSO were required before HL-60 cells were committed to differentiate (43). Heat shock is therefore an extremely effective inducer if defined in terms of time of exposure needed to commit cells to differentiate. An analysis of the rate of appearance of functions typical of cells expressing a more mature phenotype showed that the heat shock rapidly induced changes in gene expression (Fig. 2). We suggest that the variability of the period required for each agent to commit the cells to a program of differentiation may be a reflection of the rate of accumulation of sublethal damage imposed by each substance which then triggers a cellular response which leads to phenotypic change.

The finding that a heat shock induced the differentiation of HL-60 cells led to the investigation of the activity of a variety of agents which had been reported to induce the expression of hsps and/or thermotolerance (22–24, 36–38). We have reported previously that ethanol was an inducer of HL-60 cell differentiation (3) (Table 1) and like other polar solvents, such as dimethylformamide and DMSO, it has been reported to induce the expression of hsps and the thermotolerance of some cells (23, 24, 36). Each of the structurally and pharmacologically disparate agents which were studied induced HL-60 differentiation at concentrations which were marginally below those which were cytotoxic (Table 1). These results lent further support to the hypothesis that the induction of some type of stress response might be involved in the commitment of HL-60 cells to differentiation.

The imposition of a heat shock of 43.5°C induced the expression of the major hsps in these cells (Fig. 3), as did incubation of the cells for 1 h with toxic concentrations of NMF or ethanol (1 M) (*Lanes H* and *I*). When we investigated the time course of protein synthesis in HL-60 cells treated under conditions for

the optimal induction of differentiation by 170 mM NMF (3) we were surprised to observe that the constitutive synthesis of the hsps was rapidly and selectively depressed, particularly the synthesis of the M_r 90,000 protein (Fig. 3). As this manuscript was being revised, similar findings were reported for M_r 70,000 hsp synthesis in Friend erythroleukemia cells which were made to differentiate by incubation with dimethylsulfoxide (44). hsp synthesis has been shown to decrease in quiescent cells (45), and it might be considered that in the Friend cell, where DMSO brings about a transient inhibition of DNA synthesis (46), that the changes in the synthesis of hsps which were observed might have simply reflected changes in the replicative status of the cells. However, treatment of HL-60 cells with 170 mM NMF does not inhibit their replication until after 24 h of continuous treatment (47) so that we do not consider that the early (between 2 and 6 h) falls in hsp synthesis simply represent an adverse effect of NMF on cell kinetics. Moreover, preliminary evidence suggests that the constitutive synthesis of the heat shock proteins is reestablished as time progresses, albeit at lower levels.[5] The mechanism whereby low concentrations of NMF (170 mM) reduced constitutive hsp synthesis, whereas a high concentration (1 M) induced synthesis is, extremely puzzling and is currently under investigation by us. Another puzzling feature of our results is the observation that a heat shock induced significant, albeit low and variable, amounts of differentiation (Figs. 1 and 2), but, unlike an effective concentration of NMF, the heat shock induced hsp synthesis (Fig. 3). One possible explanation of this paradox may be that while heat is a strong inducer of certain hsps (hsp 70) there are others which appear to be regulated differently, for example those called hsx 70 and hsc 70 (40), or it is possible that there were temporal changes in heat shock protein synthesis during the period of heat shock, perhaps involving an initial reduction of synthesis. Our present experiments aim to resolve the questions of precisely which of the proteins of this family of proteins is being regulated under different conditions and their temporal control. Preliminary results (1) suggest that there is also temporal regulation of cellular amounts of these proteins, in addition to the rate of their synthesis, as the cells are induced to differentiate.

Recent reports have implicated the hsps in interactions with the products of certain oncogenes, acting to stabilize otherwise transient proteins such as *myc* and p53, so that the hsps may perhaps play a role in maintaining the malignant phenotype (28–30). The high constitutive synthesis of the hsps in HL-60 cells, particularly hsp 90 (see Fig. 3) may play an important role in maintaining their malignant phenotype. Our current hypothesis is that agents such as NMF may mildly denature other cellular proteins, under conditions where hsp synthesis is not stimulated, so that hsps associated with the stabilization of the products of genes responsible for the malignant phenotype are "competed off" by these modified proteins, allowing proteolysis of the oncogene protein. It is possible that this may then permit the cells to complete their programs of development. We will report on attempts to test this hypothesis in the future.

Finally, the regulation of hsp synthesis has been suggested to occur as a response to the modification of proteins, the presence of nascent unfolded proteins or the accumulation of abnormal amounts of normal cellular proteins (25–27). Inspection of the literature of the promotion of HL-60 cell differentiation suggests that all of the effective agents (see Introduction) could be argued to interact with or modify proteinaceous targets, with the possible effect of changing their normal conformation during the prolonged periods of incubation required to commit cells to terminal differentiation. Direct DNA-damaging agents, such as the alkylating agents, have not been reported to be effective as inducers of HL-60 cell differentiation. In our hands nitrogen mustard is toxic to HL-60 cells but is not an inducer of differentiation.[5] This may give clues as to the mechanism whereby so many disparate agents are able to induce the maturation of HL-60 cells.

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